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(57) Abstract

A nucleic acid sequence in a tissue section is amplified using PCR techniques: the tissue section is attached to a support structure, such as glass coverslip which may be cut to size, placed inside a PCR tube and exposed to PCR reaction components and thermal cycling in known PCR apparatus. An extract from the PCR tube is used to provide a template for manufacture of labelled probe.

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NUCLEIC ACID AMPLIFICATION AND IN-SITU DETECTION

This invention relates to nucleic acid amplification and in-situ detection, achieved by amplification of a target nucleic acid using techniques of the polymerase chain reaction (PCR) and subsequent in-situ hybridisation of a labelled probe with amplified target nucleic acid. This invention has been called "Immersion -histo PCR".

PCR is the name given to the *in vitro* amplification of a specific nucleic acid sequence by repeated cycles of primer directed synthesis. The technique is described by R. K. Saiki, et al in *Science*, 1995, **230**, 1350 and by K. B. Mullis in *Sci. Am.* 1990, **262**, 36.

PCR techniques make it possible rapidly to detect trace amounts of any known DNA or mRNA sequence in tissue extracts. However, one major limitation of the technique is that it is performed in solution and the results of PCR can not be correlated with cell types in the tissue. A methodology, referred to as in-situ PCR, has been developed to overcome this limitation. This combines the amplification power of PCR and the precise localisation of in-situ hybridisation for nucleic acid sequences within individual cells in tissue sections.

Several protocols of in-situ PCR on glass mounted tissue sections has been described. All of them exhibit major problems related inter alia to poor sensitivity, poor reliability and poor specificity - largely due to inaccurate temperature control and evaporation of reaction mixture.

An in-situ PCR amplification system is described in EP-A-0611598. This system discloses that a complete in-situ PCR system requires a glass microscope slide, a specimen sample containing the target nucleic acid sequence, a flexible plastic cover that fits over the sample and a retaining assembly fastened to the slide and to the cover to retain and seal a reaction mixture against the sample during PCR thermal-cycling. The retaining assembly includes a relatively complex mechanism

to retain the plastic cover (seal) over the slide.

This system presents difficulties and disincentives to the user. The system is relatively expensive, especially when it is borne in mind that a typical laboratory would want to carry out many hundreds of in-situ PCR reactions. The specialised machine designed to work with the system accommodates only a limited number of in-situ PCR systems. Further, the combination of the microscope slide, plastic cover and its retaining assembly reduces the efficiency of temperature control and therefore reduces the efficiency of thermal-cycling needed for the PCR reaction to proceed. The presence of the plastic cover makes some standard PCR techniques awkward to utilise, for example semi-nested PCR is not routinely possible using this system. Finally, the system has been designed for the amplification stages of in-situ PCR, meaning that later stages of the detection process, such as in-situ hybridisation, are not incorporated into the system.

A second in-situ PCR system, again using dedicated machines, has been developed by Hybaid, and termed the OmniGene *In*-situ system (trade mark of Hybaid Limited). This system uses a similar slide plus seal arrangement to that described in EP-A-0611598 and allows access to the PCR reaction by providing a plastic cover comprising a reaction chamber that can be opened. This system, however, suffers from similar disadvantages to the system described above, that is to say that temperature control is inefficient, so thermal-cycling can not be performed as conveniently as for in solution PCR. The system also proposes three separate dedicated pieces of equipment, thus taking the system out of the financial capacity of many small laboratories.

As a result, some workers in this field wishing to carry out in-situ PCR still use a technique developed by Nuovo et al and described in American Journal of Pathology, 139, #6, pages 1239-1244 (1992). This technique covered the tissue sections with a coverslip, anchored using nail polish. Again, thermal-cycling is inefficient using this method but quite simply it is the only low cost method available to workers in this field.

There thus exists a pressing need for the development of an improved in-situ PCR method that will enable amplification of a target nucleic acid sequence and its in-situ detection.

It is an object of this invention to provide an improved and preferably simplified procedure for carrying out in-situ PCR and for detecting PCR products in-situ. It is a further object to provide a method for localisation of PCR product in a tissue sample. A still further object is to enable efficient carrying out of in-situ PCR methods and to enable generation of a labelled probe for carrying out in-situ hybridisation. Yet another object of the invention is to remove or at least ameliorate the problems identified in prior art procedures.

According to the invention, a method of amplification of a specific nucleic acid sequence, which sequence is located in a tissue section, comprises attaching the tissue section to a support structure to form a composite, immersing the composite in a solution adapted for carrying out polymerase chain reaction (PCR) and amplifying the sequence by PCR.

In use, a tissue section is attached to a support structure, such as a rigid glass coverslip or a flexible sheet of plastics material, using a suitable adhesive, to form a composite. In the case of a flexible support it may then be possible to fold up the composite or roll up the composite or otherwise compact the composite so that it can fit inside a container in which the PCR reaction will be carried out. In the case of a rigid support it might first be necessary to cut down the composite to a suitable size to be placed inside a container in which the PCR reaction will be carried out. Prior to PCR amplification, the tissue section is typically de-waxed according to standard procedures and is further typically subjected to a proteinase digestion step. The section is subsequently immersed in a solution containing PCR reagents and subjected to PCR amplification, comprising thermal cycling and if necessary introduction of further reagents into the solution, according to conventional PCR techniques. One surface of the tissue section is adhered to the support structure and the other surface is left exposed to the PCR solution in which

the composite is immersed.

The composite may be sufficiently small to go inside a conventional 0.5ml PCR tube, in which case the PCR amplification can be carried out using standard PCR apparatus. Alternatively, the composite may be larger and may then be placed inside a vial containing a greater volume of PCR solution, and the PCR amplification, including the stages of thermal cycling, carried out in suitable water baths.

In accordance with the invention, a method for in-situ identification of a target nucleic acid sequence is provided, comprising carrying out amplification of the target nucleic acid in a PCR tube. This offers the advantage that the PCR amplification stage is carried out using conventional and well known solution PCR techniques. The PCR amplification step can thus include semi-nested procedures if desired. In the techniques described in EP-A-0611598 semi-nested procedures were not possible. Using the system described by Hybaid Limited, semi-nested PCR was rather awkward as reaction components had to be pippetted into the small coverslip reaction chamber. Using conventional solution PCR, semi-nested reaction components are simply added to the PCR tube in the normal way.

Conventional PCR techniques are well known in the art and are described in EP-A-0201184 and EP-A-0200362. The term "PCR techniques" is intended to refer to the techniques described in these two publications.

The method of the invention can conveniently be carried out by performing target nucleic acid amplification in a conventional PCR tube having a capacity of between 0.1ml and 2.0ml. Known PCR tubes that are useful in the method of the invention include tubes made of plastics material having an integral cap that enables the tube to be opened and re-sealed any number of times. These tubes typically have a volume of between 0.2ml and 1.0ml, commonly 0.5ml.

Most laboratories, even the smallest ones, include a PCR machine and a supply of

conventional PCR tubes, so the method of the invention enables in-situ PCR to be carried out conveniently and cheaply by all those currently using conventional in solution PCR.

In a specific embodiment of the invention, described in an example below, a tissue section is attached to a support surface, such as a glass coverslip for a microscope slide, forming a composite, the tissue-support composite is then cut to a piece small enough for its introduction into a 0.5ml standard PCR tube, the piece is placed in a tube and PCR techniques are performed substantially as for conventional solution PCR. For in-situ hybridisation, a labelled probe is added to the same PCR tube. The tissue section is then mounted and examined, again according to the conventional procedures.

In preferred use of the invention, a tissue section is mounted on a support surface and then de-waxed. De-waxing may be carried out using xylene for a period typically of around 8 minutes and according to standard procedures. Xylene is then washed from the tissue section using alcohol and the section is then rehydrated. This is again a standard procedure and alternative methods of preparing the section are well known. Rehydration can be achieved with a series of washing steps using alcohol of decreasing concentration and ending with an aqueous solution such as buffer. Alternatively, the section can merely be rinsed in alcohol and allowed to dry. A further optional, though preferred, step is to subject the tissue section to a proteinase digestion step, prior to amplification of the nucleic acid by PCR techniques. Including this digestion step can facilitate access of PCR reagents to cells in the section and increase the retention of amplified nucleic acid within target cells, giving a greater signal following in-situ hybridisation with labelled probe. Suitable proteolytic enzymes are proteinase K, trypsin, pepsin and other proteinases.

The period of proteinase digestion is adjusted according to the particular tissue section. The proteinase digestion is to enable access of PCR reagents to nucleic acid target inside cells in the section. In the absence of digestion, or if the

digestion is insufficient, then the considerable cross linking of proteins and other macromolecules in the tissue section typically prevents PCR reagents from diffusing into cells. On the other hand, if proteinase digestion is excessive then PCR product will readily leak out of the cell in which the target sequence is found, will subsequently diffuse into surrounding cells and tissues and give excessive background signal.

It is preferred for the PCR amplification step to comprise the use of primers specific for amplification of the target sequence and also to comprise blocking primers. The blocking primers are typically present at a ratio of blocking: specific primer of up to 1:2, preferably up to 1:5. The blocking primers are adapted to amplify a sequence found in most of the cells in the tissue section, and preferably found in virtually all or all of the cells in the tissue section. An example is a set of primers suitable to amplify a sequence from the beta-globin gene. Other suitable sequences to be amplified by the blocking primers are any repetitive sequence, the Alu sequence or a microsatellite sequence. Primers for these sequences are readily available in the market place.

When the method of the invention is performed using both specific primers and blocking primers, reduced background noise is observed. It is possible that amplification of non-target sequences in many cells of the section prevents diffusion into these cells of amplified target sequence from the few cells in which the target sequence is located.

According to a further aspect of the invention there is thus provided a kit of primers suitable for use in in-situ PCR, wherein the kit comprises a first subset of primers adapted for PCR amplification of a first sequence and a second subset of primers adapted for PCR amplification of a second sequence, wherein the first subset of primers is adapted to amplify a sequence that is to be subsequently identified and/or localised in a tissue section by in-situ hybridisation and the second subset of primers is adapted to amplify a sequence that is common to cells in the tissue section, and wherein the ratio of the respective subsets of primers is 2:1, or

greater.

The ratio of the respective subsets of primers is preferably at least 5:1 and more preferably at least 10:1. In a specific embodiment of the invention, a kit of primers comprises a first subset of primers adapted to amplify a portion of an EBV sequence and a second set of primers adapted to amplify a beta-globin sequence, and wherein the ratio of the first:second subsets of primers is about 10:1. The primers for amplification of the beta-globin sequence act as blocking primers in that in use during in-situ PCR, amplification of the EBV sequence is achieved using the first subset of primers, generating an amplified EBV sequence, and this amplified sequence is inhibited from diffusing into neighbouring cells as in those neighbouring cells there has been a certain amount of amplification of the beta-globin sequence using the second subset of primers.

An optional, additional step in the method of the invention is to use PCR product from the amplification steps to generate a labelled nucleic acid probe (DNA or RNA). This can conveniently be achieved by extracting an aliquot of reaction mixture from the PCR tube, using the contents of this aliquot to provide a template for manufacture of a labelled probe, in the presence of labelled nucleotides or nucleosides, and subsequently using said labelled probe to carry out in-situ hybridisation with the target nucleic acid for identification and localisation thereof. In-situ hybridisation is a widely known and used procedure, consisting essentially of providing a labelled probe that is homologous with or substantially homologous with a portion of or the whole of the target nucleic acid. The probe is added to a solution in contact with a tissue section, the section is washed in the same tube and then examined for successful hybridisation. Where hybridisation occurs this indicates that the target nucleic acid was indeed present in the tissue section and the precise location of the nucleic acid can be identified as successful hybridisation can be identified in individual cells.

Labelled probe is alternatively prepared using PCR product from a positive control.

The invention thus renders in-situ PCR a practical test. The method of the invention has been termed "immersion-histo PCR" (IHPCR) by the inventor. The invention typically utilises coverslips to replace glass slides for support of the tissue sections. Being of relatively thin width, the coverslip allows rapid heat transfer and does not hinder the thermal cycling required for PCR. The coverslips together with the tissue section can conveniently be cut into a smaller size to be placed into a PCR micro-tube. The DNA or RNA (after reverse transcription) sequence of interest in the tissue section is then amplified using routine PCR methods. Some of the products inevitably diffuse into the reaction mixture and these, or alternatively the products of a positive control, can be used as template for a second round of PCR to generate a labelled DNA probe, for example using digoxigenin-labelled dUTP. The tissue sections are washed and the amplified DNA sequences detected by hybridisation with the PCR-generated probe in the same micro-tubes. Since DNA amplification can be carried out in a micro-tube in an ordinary PCR cycler, the invention enables the performance of in-situ PCR comparable to that of conventional solution PCR and greatly improves the reliability, sensitivity, specificity and flexibility for amplification and detection of cellular DNA or mRNA sequences of interest in tissue sections compared to prior art in-situ techniques.

In an embodiment of the invention there is provided a method for in-situ identification of a target nucleic acid sequence, comprising:-

creating a reaction composite by attaching a tissue section to a surface,

placing the reaction composite inside a vial,

amplifying the target nucleic acid in the vial using polymerase chain reaction techniques, and

identifying amplified target nucleic acid by in-situ hybridisation with a labelled nucleic acid probe.

The tissue section can be attached to the surface by first cleaning or washing the surface and then using a suitable adhesive to attach the tissue section thereto. One conventionally used adhesive for this purpose is 3-aminopropyltriethoxy-silane (AMPES).

The PCR amplification step is carried out in solution and using reaction components and conditions that are conventional for solution PCR, the components typically including primers, DNA polymerase, dATP, dCTP, dGTP, dTTP and polymerase buffer.

The surface is preferably a substantially planar surface of a thin sheet of glass or plastics or composite material. Particularly suitable for laboratories is a glass or plastic coverslip. A standard size coverslip commonly found in laboratories is about 22mm x 22mm; another is about 22mm x 26mm. The coverslip is thin and allows more rapid heat transfer between the tissue section and the surrounding solution than is possible using the thicker glass slides adopted in prior art in-situ PCR procedures. Once the tissue section is attached to the surface, such as the surface of a coverslip, then it is convenient to cut out a smaller piece that will fit into a conventional PCR tube. A known PCR microtube is a 0.5ml microtube, with length about 30mm and diameter (at opening) about 8mm. A convenient size of cut coverslip to go inside a PCR microtube is 3-4mm x 5-15mm. Typically, a coverslip can be cut into a piece approximately 3mm by 5mm, a convenient size to go inside a PCR tube.

Using forceps or similar apparatus to hold a coverslip to which a tissue section is attached, a small piece can be cut away using a diamond pen to generate a piece of suitable size to go inside a PCR tube.

Accordingly, a further embodiment of the invention comprises the steps of:-

making a reaction composite by attaching a tissue section to a support surface, said support surface being a substantially planar surface of a sheet

of glass or plastics or composite material,

separating out a portion of said reaction composite, said portion being sufficiently small to fit inside a PCR tube,

placing said portion in said PCR tube,

amplifying the target nucleic acid using PCR techniques, and

detecting amplified target nucleic acid sequence in-situ by in-situ hybridisation.

The portion of the reaction composite can be adapted to fit inside a chosen PCR tube by cutting away or otherwise removing other portions of the reaction composite, the portion going in to the tube necessarily comprising a portion of the tissue section and a portion of the support surface, such as a portion of the coverslip.

The target nucleic acid is amplified using PCR techniques, such as by adding to the tube suitable buffer, primers, DNA polymerase and nucleoside triphosphates. As already discussed above, these techniques will be well known to a person of skill in the art. A suitable PCR primer, for example, has about 20 bases, commonly between 16 and 35 bases. According to these known techniques, the target nucleic acid sequence, where it is present, is amplified by thermally cycling the contents of the PCR tube.

An optional additional step is to extract from the PCR tube an aliquot containing amplified target nucleic acid sequence. This sequence is then used as a template, for further PCR amplification in the presence of at least one labelled nucleoside or nucleotide, to form a labelled probe. A typical probe length is in the range 100-400 bases or base pairs. This labelled probe can then be used to carry out in-situ hybridisation on the tissue section to localise the amplified target nucleic acid. As

will be appreciated by persons of skill in the art, this additional step enables the convenient manufacture of a suitable probe to complete the in-situ PCR without the need for purchase or separate manufacture of a probe. Simply, a suitable probe can be made as a by-product of the in-situ PCR method. Lastly, the probe is preferably made using inner primers in a nested PCR reaction.

Accordingly, the invention also provides a method of making a labelled probe, for use in in-situ hybridisation, comprising

identifying a tissue section containing the probe sequence;

attaching said tissue section to a support surface to form a composite;

cutting said composite down to a size sufficiently small to go into a PCR tube;

amplifying the probe sequence using PCR techniques to make labelled probe.

Various advantages of the method of the invention will be apparent. The invention eliminates the need for the special machines, glass microscope slides, dedicated seals and other apparatus advocated in the prior art. The method makes available to all laboratories with an ordinary PCR cycler the ability to carry out in-situ PCR. The use of positive PCR products as a template to generate a probe makes it possible to detect any known nucleic acid sequence within a cell on a tissue section without worrying about the availability of a suitable probe. A specific embodiment of the invention examined the methods of invention in four different cases.

In case 1 (EBV-associated large B-cell lymphoma), tumour cells were clearly delineated by intense nuclear staining for PCR amplified EBV BamHI W fragment. This staining pattern matched that of in-situ hybridization for EBV EBER RNA. Strong nuclear staining for t(11;14) and t(14;18) was observed in over 60% of tumour cells

in the mantle cell (case 2) and follicular lymphoma (case 3) respectively. In case 2, the staining pattern was closely similar to that of immunostaining for cyclin D1, which highlights tumour cells. In case 3, the distribution of the t(14;18) positive cells closely resembled that of CD20 positive B-cells. Moderate to strong staining for clone-specific IgVH fragment was noted in over 60% of tumour cells in the large cell B-cell lymphoma, while small lymphocytes were negative. In all cases endothelial cells and other non-lymphoid cells were negative, with the exception of macrophages which sometimes showed weak granular cytoplasmic and nuclear staining. Discrete PCR products of the expected size were identified in the solution phases of the IH-PCR reactions by polyacrylamide gel electrophoresis. Co-amplified control sections of tonsil and unrelated lymphomas showed weak staining in macrophages and occasional cytoplasmic positivity in small lymphocytes, which was greatly reduced by optimization of protease digestion.

Controls without primers, without Taq polymerase, and with unrelated primers were negative in cases 2 to 4. In case 1 (EBV associated lymphoma) weak granular nuclear staining was seen in some of the tumour cells in each control following hybridization with the EBV BamHI W probe.

In the absence of proteinase k digestion, weak staining was observed in the target cells in all cases after IH-PCR. The staining intensity and the amounts of PCR products in solution increased with increasing concentration of proteinase K, up to 10 μg/ml, but at higher concentrations specific signal was progressively lost. Staining in non-target cells appeared at concentrations above 5 μg/ml. At 20 μg/ml intra-cellular signal was lost, although specific products in the solution phase were clearly seen on gels. Optimal proteinase K concentrations giving high specific signal with minimal staining of non-target cells were found to be restricted within a narrow range of 3-5 μg/ml, at which concentrations PCR products in the solution phase were only weakly seen.

Negative control tonsil sections co-amplified using t(11;14) primers in the presence of case 2 showed no staining at enzyme concentrations below 5 μg/ml. At higher

proteinase k concentrations staining of increasing intensity was seen in all tissue components together with consistently strong specific staining of the lymphoma.

Specificity of PCR amplification according to the invention was confirmed by gel analysis of solution phase products. Signal representing EBV W DNA fragment was amplified by IH-PCR to levels comparable to those of in-situ hybridization for EBV EBER RNA which is present in thousands of copies in each infected cell. Cells carrying positive signal for the chromosome translocations and Ig sequences were shown to be tumour cells by their cytology and immunostaining pattern. For detection of single copy genomic DNA sequences, no signals were generated in control reactions without specific primers, Taq polymerase, or with unrelated primers. The flexibility of the technique also allowed further examination of cross-reactivity by simultaneous target amplification and signal detection of two unrelated tissue sections in the same tubes. The negative results in non-target cells of the same section and co-amplified control sections suggest that staining by IH-PCR was specific.

Fixation of sections with paraformaldehyde after protease digestion and after amplification, and dehydration with ethanol before and after amplification are convenient in immobilizing amplified products and minimizing staining of non-target cells. In addition, it is optional to incorporate non-target (e.g. β-globin) primers in the IH-PCR mixtures to reduce non-specific staining of cells. These may be referred to as blocking primers. It is presumed that amplifying sequences which are potentially present in all cells and are unrelated to the probe used, blocks amplification of target genes and adherence of products following diffusion into non-target cells. However, as high concentrations of β-globin primers are found to reduce specific signal, a ratio of specific primers to blocking primers of about 10:1 is employed in examples below. The ration of specific primers to blocking primers (when present) is suitably at least 2:1, preferably at least 5:1.

The reaction conditions for IH-PCR are generally similar to those for solution PCR. Optimal $MgCl_2$ concentrations were between 3 and 4 mM, whereas those for solution PCR were 1 to 1.5 mM. Sequestration of magnesium by the glass slide and the

tissue section may decrease the effective concentration. However, in IH-PCR, at low concentrations of $MgCl_2$ (1-2 mM), abundant PCR products were observed in the solution phase, while very low signals were seen in the target cells. Increasing the concentration of $MgCl_2$ led to increase of specific signal, but resulted in significant reduction of products in the solution phase. These findings suggest that higher $MgCl_2$ concentrations per se may be required for efficient amplification within cells in tissue sections.

PCR-generated probes offer several advantages over either oligonucleotide or genomic probes. They allow flexibility of design to give optimal size and target specificity. The probes can be easily prepared using PCR products as templates, eliminating the requirement for commercial probes. For demonstration of exogenous (viral) DNA sequences, the same primers for amplification of the target in the sections have been successfully used for probe generation. But, for complex genomic DNA, nested or semi-nested primers for probe generation may be required to minimize non-specific signal. Carrying out the in-situ hybridization procedure in small volumes of reagents in micro-tubes is economical and simplifies the process.

According to a second aspect of the invention there is provided a kit for carrying out in-situ amplification of a target double stranded nucleic acid and for identification thereof using in-situ hybridisation, the kit comprising:-

at least two oligonucleotide PCR primers, adapted to be substantially complementary to respective ends of respective strands of the target nucleic acid sequence and

a labelled nucleic acid probe adapted to be substantially homologous with a portion of or the whole of a PCR product generated by the PCR primers.

This kit is of advantage to the user of in-situ PCR techniques in that the development and testing of optimal primers and optimal probes can take a considerable amount of time. By providing primers and probe in a single kit, in-situ PCR can rapidly used

by any person of skill in the art without the need to spend time developing one's own primers and probes.

A description of a specific embodiment of the invention now follows accompanied by the drawings in which:-

Figs. 1-3 show the results of in-situ nucleic acid amplification and detection using primers to amplify a sequence from Epstein Barr Virus (EBV); and

Figs. 4-6 shows the results of in-situ nucleic acid amplification and detection using primers to amplify a sequence found in cells in which a chromosomal translocation (t(14;18)) has occurred;

Figs. 7-10 shows the results of in-situ PCR for EBV associated large B-cell lymphoma;

Figs. 11-19 show the results of in-situ PCR for visualisation of single copy allele specific DNA sequences in lymphomas with PCR generated probes; and

Fig. 20 is a schematic diagram of positions of the primers used for IH-PCR and probe generation.

Example 1

A tissue section of choice is attached to a coverslip and cut into an appropriate size using a diamond pen.

The cut down coverslip with cut down tissue section attached is then transferred using forceps to a PCR micro-tube. Optional tissue section treatments, such as digestion and post-digestion fixation can be performed in this micro-tube.

PCR amplification of the target nucleic acid, if present, is carried out in a routine PCR

machine using conventional PCR techniques. Optionally, PCR techniques such as semi-nested PCR can be performed conveniently.

To detect whether any target nucleic acid is present and to localise amplified target nucleic acid to a particular cell or other part of the tissue section, the next step is to perform in-situ hybridisation. This is performed using a labelled probe, optionally generated using a template that is a PCR product obtained in the same micro-tube.

After in-situ hybridisation the tissue section with coverslip is washed and mounted on to a glass slide for subsequent visual microscopic and other examination to determine the localisation of the amplified nucleic acid sequence.

Example 2

In-situ PCR was carried out to test for the presence of EBV in a tissue section.

The primers used were:

(1) 5' AAA GCG GGT GCA GTA ACA GCT AAT (SEQ ID NO: 1)
(2) 5' TTG ACT GAG AAG GTG GCC TAG CAA (SEQ ID NO: 2)

These primers were designed to amplify the W fragment of the virus and were used to generate PCR labelled probe.

The results are shown in Figures 1-3, where Fig. 1 shows a control tissue section without PCR amplification hybridised with PCR generated EBV W fragment probe, Fig. 2 shows a control section amplified without primers hybridised with the same probe, Fig. 3 shows a section showing amplified EBV (dark staining) in infected cells; thus 1 and 2 show that, without in-situ PCR amplification, no target DNA is detectable. 3 shows that following PCR amplification using these two primers, target DNA is detectable, by in-situ hybridisation with labelled probe generated using the primers.

Example 3

In-situ PCR was carried out to detect t(14;18) chromosomal translocation.

The primers used were:

(1) 5' TTA GAG AGT TGC TTT ACG TG (SEQ ID NO: 3)

(2) 5' ACC TGA GGA GAC GGT GAC CAG GGT (SEQ ID NO: 4)

These primers were for the bcl-2 and JH genes, and were also used for probe generation.

The results are shown in Figures 4-6. Fig. 4 shows a control tissue section without PCR amplification hybridised with PCR generated bcl-JH probe, fig. 5 shows a control section amplified without primers hybridised with the same probe, and Fig. 6 shows a section showing amplified translocated DNA fragment (dark staining) in lymphoma cells; thus 4 and 5 shows that no target DNA was detectable. 6 shows that target DNA is detectable in selected cells, in this case identifying lymphoma cells containing the translocation.

Example 4

Tissue samples and target genes. Formalin-fixed, paraffin-embedded lymph node blocks were selected from the archives of the Histopathology Department at University College London Medical School. These included a reactive tonsil, a mantle cell lymphoma known to contain t(11;14) translocation, a follicular lymphoma known to contain t(14;18) translocation and two large B-cell lymphomas (one EBV associated). The chromosomal translocations were originally detected by PCR and further confirmed by sequence analysis. Sequence data of the clonally rearranged IgVH gene in the non-EBV associated large B-cell lymphoma were available from a previous study (*Blood* **87**, 2428-2434 (1996)). Sections of appropriate cases routinely immunostained for CD20 and cyclin D1 or hybridized for demonstration of EBV EBER

RNA were also available for comparison.

Primer selection. Primer design was based on sequences from the Genbank database or sequences derived from relevant cases (Table 1 below). Suitability of primer sets for specific amplification of target genes for IH-PCR and probe generation were determined using solution phase PCR and those producing high yield with minimal non-specific products were selected. The relative positions of primers for IH-PCR and probe generation are shown in Fig. 20.

Section preparation and pre-treatment. Four μm sections were mounted on APES (Merck, UK) coated coverslips and dried at 55 °C overnight. Sections were de-waxed in xylene, washed twice in ethanol and dried at room temperature. The coverslips were cut into pieces, 3 to 5 mm in width, using a diamond pen and a pair of forceps. The sections were incubated at 37 °C in 0.5 ml micro-tubes in a solution containing 0.1 M Tris-HCL (pH 8), 50 mM EDTA and from 0 to 20 μg/ml (see below) of proteinase K for 30 min. Sections were treated with 4% paraformaldehyde/PBS for 10 min in the same tubes. After washing twice with PBS and twice with ethanol, the tissue fragments were dried at room temperature.

IH-PCR. Pre-treated sections of cases 1 to 4 were placed back to back with pre-treated control sections of reactive tonsil and/or unrelated lymphomas in 0.5 ml micro-tubes with 46 μl of reaction mixtures containing 0.5 μM each target gene primer (Table 1), 0.05 μM each blocking primer (β-globin), 200 μM each dNTP, 2-4 mM $MgCl_2$ and 1 X PCR buffer (Promega, Southampton, UK). Reaction mixtures were overlaid with mineral oil and heated to 93 °C for 3 min in a thermal cycler (Hybaid, UK). The tubes were then held at 55 °C and 0.5 U Taq polymerase (Promega, Southampton, UK) in 4 μl water added. Thirty to 40 cycles of 93 °C for 40s, 55 °C for 45s and 72 °C for 110s were performed with a final extension step of 10 min. PCR products in the solution phases were analyzed on 6 or 10% polyacrylamide gels. Reactions without primers, without Taq enzyme, with unrelated primers and with one specific primer and one unrelated primer were carried out as negative controls for each case.

PCR generation of probes. Nested PCR was used to generate specific probes for each target using products (diluted 1/1000) from the original amplifications as templates (Table 1, Fig. 20). Fifty μl reaction mixtures were composed of 0.5 μM of each primer, 200 μM of dATP, dCTP and dGTP, 67 μM of dTTP, 33 μM of digoxigenin labelled dUTP (Boehringer Mannheim, East Sussex, UK), 1.5 mM $MgCl_2$, 1 X PCR buffer, 2 μl diluted PCR product and 1 unit Taq polymerase. PCR was performed for 35 cycles of 94 °C for 40s, 55 °C for 45s and 72 °C for 110s. Four μl of PCR products were analyzed on 6 or 10% polyacrylamide gels to confirm specificity and to check yield. PCR products were used directly or purified by ethanol precipitation and stored at -20 °C.

***In-situ* hybridization.** After IH-PCR each pair of the control and test sections were transferred to a clean micro-tube and post-fixed in 4% paraformaldehyde/PBS for 10 min. Sections were washed once with TBS and twice with ethanol and dried at room temperature. Fifty μl of hybridization solution containing 2 μl PCR generated probe, 50% formamide, 2 X SSC, 10% dextran sulphate, 0.25% BSA, 0.02% Ficoll 400, 0.25% polyvinyl pyrrolidone-360 (PVP, Merck, Leicestershire, UK), 0.25 M Tris-HCL (pH 7.5), 0.5% sodium pyrophosphate, 0.5% SDS and 250 μg/ml denatured salmon sperm DNA were added to each tube. The tubes were placed on a hot block at 92 °C for 8 min and then incubated at 42 °C for 6-16 h.

After hybridization the sections were washed 3 times in TBS for 5 min and incubated twice in alkaline phosphatase-conjugated sheep anti-digoxigenin (1:500 dilution in TBS, Boehringer Mannheim, East Sussex, UK) for 1 h. After 3 washes in TBS, the label was visualized with NBT/BCIP (Gibco BRL, Paisley, UK). The sections were counterstained briefly with Mayer's Haematoxylin and mounted with crystal mountant (Biomeda Corp, Foster City, CA, USA).

Optimization of protease digestion. Initial experiments and previous work have shown that the extent of proteinase k digestion can affect staining patterns following gene amplification of tissue sections. Consequently, for initial optimization, proteinase k concentrations of 0, 1, 5, 10, 15 and 20 μg/ml were carried out in each case. To

investigate uptake of PCR products by non-target cells more precisely, sections of reactive tonsil digested with 1, 5, 10, 15 and 20 μg/ml proteinase k were co-amplified in the same tube with digested (10 μg/ml) sections of case 2 (mantle cell lymphoma). Sections were subjected to IH-PCR using t(11;14) primers and stained as above.

Figs. 7-10 show Case 1, EBV associated large B-cell lymphoma. EBV BamHI W DNA fragment was visualized by IH-PCR with a PCR generated probe. Fig. 7, Control IH-PCR without primers shows weak granular nuclear staining in some of the tumour cells. Fig. 8, After IH-PCR, the majority of tumour blasts have condensed nuclear staining, while endothelial cells are negative. Fig. 9, Standard in-situ hybridization for EBER RNA shows strong signals in tumour cells. Fig 10, Tonsil section co-amplified with Fig. 8 shows no signals in lymphoid cells, but occasional weak staining in tingible body macrophages.

Figs. 11-19 show IH-PCR visualization of single copy allele specific DNA sequences in lymphomas with PCR-generated probes. Fig. 11, Immunostaining (peroxidase/DAB) for cyclin D1 shows moderate staining in the tumour cells of case 2, mantle cell lymphoma. Fig. 12, IH-PCR for t(11,14) translocation demonstrates strong nuclear staining in the tumour cells of the same case. Fig. 13, Tonsil section co-amplified with Fig. 12 is negative. Fig. 14, Tumour cells in case 3, follicular lymphoma show strong immunostaining for B-cell marker, CD 20. Fig. 15, Tumour cells in case 3 are clearly delineated by strong nuclear staining after IH-PCR for t(14;18). Fig. 16 Except for weak staining in some tingible body macrophages, co-amplified tonsil section is negative. Fig. 17, Tumour cells in case 4, large B-cell lymphoma are highlighted by immunostaining for CD20. Fig. 18, IH-PCR for allele specific IgVH shows moderate nuclear staining in large tumour cells, whereas non-tumour cells are negative. *i*, Tonsil section co-amplified with Fig. 19, shows a negative result.

Fig. 20 is a schematic diagram showing positions of the primers used for IH-PCR and probe generation.

The invention has thus provided a simple and low cost method for in-situ PCR. Other

examples and embodiments of the invention will be apparent to persons of skill in the art.

Table 1. Primer Sequences for IH-PCR and Probes

Case	Diagnosis	Target Locus /Gene	Code and Sequence (5'-3')		Target/Probe Size
1	EBV-LCL	EBV BamHI W	*EBV* OUTER 1-1:	AAAGCGGGTGCAGTAACAGGTAAT (SEQ ID NO: 1)	1-1 to 1-2 (Target): 315 bp
			EBV OUTER 1-2:	TTGACTGAGAAGGTGGCCTAGCAA (SEQ ID NO: 2)	1-3 to 1-4 (Probe): 200 bp
			EBV INNER 1-3:	GATTTGGACCCGAAATCTGA (SEQ ID NO: 5)	
			EBV INNER 1-4:	TCTGGGGGCTTATTCCTCTT (SEQ ID NO: 6)	
2	MCL	t(11;14) - (*bcl-1/IgJH*)	*Bcl-1* OUTER 2-1:	GGATAAAGGCGAGGAGCATA (SEQ ID NO: 7)	2-1 to 2-2 (Target): 218 bp
			IgJH 2-2:	ACCTGAGGAGACGGTGACCAGGGT (SQ ID NO: 4)	
			Bcl-1 INNER 2-3:	ATTGCTGCACTGCATATTCG (SEQ ID NO: 8)	2-3 to 2-2 (Probe): 198 bp
3	FL	t(14;18) - (*bcl-2/IgJH*)	*Bcl-2* OUTER 3-1:	TTAGAGAGTTGCTTTACGTG (SEQ ID NO: 3)	3-1 to 3-2 (Target): 174 bp
			IgJH INNER 3-2:	TCCCTGGCCCCAGGGGTCGA (SEQ ID NO: 9)	
			Bcl-2 INNER 3-3:	GCCTGTTTCAACACAGACCC (SEQ ID NO: 10)	3-3 to 3-2 (Probe): 154 bp
4	LCL	IgVH	CDRII 4-1:	TTTATAAGGACTCTGTGCAG (SEQ ID NO: 11)	4-1 to 4-2 (Target): 176 bp
			CDRIII-CSP 4-2:	CCGTATTTATGTTGGGCAGT (SEQ ID NO: 12)	
			FR3 4-3:	ACACGGCC/TC/GTGTATTACTGT (SEQ ID NO: 13)	4-3 to 4-2 (Probe): 93 bp

Note: 1. EBV-LCL - EBV associated large B cell lymphoma; MCL - Mantle cell lymphoma; FL - Follicular lymphoma;

2. CSP - clone specific primer; Except for EBV, the primer combinations may not be applicable to all equivalent targets.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: University College London

(B) STREET: 5 Gower Street

(C) CITY: London

(E) COUNTRY: GB

(F) POSTAL CODE (ZIP): WC1E 6BT

(A) NAME: PAN; Langxing

(B) STREET: 27 Chester Road, Canning Town

(C) CITY: London

(E) COUNTRY: GB

(F) POSTAL CODE (ZIP): E16 4NL

(ii) TITLE OF INVENTION: Nucleic Acid Amplification And In-Situ Detection

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9516636.9

(B) FILING DATE: 14-AUG-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAAGCGGGTG CAGTAACAGC TAAT 24

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTGACTGAGA AGGTGGCCTA GCAA 24

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTAGAGAGTT GCTTTACGTG 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACCTGAGGAG ACGGTGACCA GGGT 24

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATTTGGACC CGAAATCTGA 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTGGGGGCT TATTCCTCTT 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGATAAAGGC GAGGAGCATA 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATTGCTGCAC TGCATATTCG 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCCCTGGCCC CAGGGGTCGA 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCCTGTTTCA ACACAGACCC 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TTTATAAGGA CTCTGTGCAG 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCGTATTTAT GTTGGGCAGT 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ACACGGCCTC GTGTATTACT GT 22

CLAIMS

1. A method for amplification of a specific nucleic acid sequence, which sequence is located in a tissue section, comprising attaching the tissue section to a support structure to form a composite, immersing the composite in a solution adapted for carrying out polymerase chain reaction (PCR) and amplifying the sequence by PCR.

2. A method according to Claim 1 wherein amplification of the sequence is carried out in a PCR tube of capacity between 0.1ml and 2.0ml.

3. A method according to Claim 1 or 2 comprising carrying out amplification of the desired nucleic acid sequence in the presence of (i) a first subset of primers specific for the desired sequence and adapted to amplify the desired sequence and (ii) a second set of primers that are specific for a sequence other than the desired sequence and are adapted to amplify the other sequence.

4. A method according to any of Claims 1-3 further comprising:-

extracting an aliquot of reaction mixture from the PCR solution,

using the contents of said aliquot to provide a template for manufacture of a labelled nucleic acid (such as DNA) probe, and

subsequently using said labelled probe to carry out in-situ hybridisation with the target nucleic acid for identification and localisation thereof.

5. A method according to any of Claims 1-4 for in-situ identification of a target nucleic acid sequence, comprising:-

creating a reaction composite by attaching a tissue section to a support surface,

placing the reaction composite inside a vial,

amplifying the target nucleic acid in the vial using polymerase chain reaction techniques, and

identifying amplified target nucleic acid by in-situ hybridisation in the vial with a labelled probe.

6. A method according to Claim 5 wherein the reaction composite is made by attaching a tissue section to a support surface and cutting out therefrom a portion of the reaction composite that is sufficiently small to be placed inside a PCR tube.

7. A method according to any of Claims 1-6 comprising the steps of:-

making a reaction composite by attaching a tissue section to a support surface, said surface being a substantially planar surface of a sheet of glass or plastics or composite material,

separating out a portion of a reaction composite, said portion being sufficiently small to fit inside a PCR tube,

placing said portion in said PCR tube,

amplifying the target nucleic acid in said PCR tube using PCR techniques, and

detecting amplified target nucleic acid sequence by in-situ hybridisation.

8. A method according to Claim 7 wherein a portion of the reaction composite is adapted to fit inside the PCR tube by cutting away or otherwise removing other portions of the reaction composite.

9. A method according to any Claim preceding wherein the target nucleic acid is amplified by adding to the PCR solution suitable buffer, oligonucleotide primers, DNA polymerase and nucleoside triphosphates.

10. A method according to any preceding Claims comprising thermally cycling the contents of the PCR tube to amplify the target nucleic acid sequence.

11. A method according to any preceding Claim comprising treating the tissue section with a proteolytic enzyme prior to PCR amplification.

12. A method for amplification of a specific nucleic acid sequence and its detection in-situ comprising:

(a) attaching a tissue section to a glass coverslip using an adhesive to form a composite;
(b) dewaxing the tissue section;
(c) placing the composite in solution in a PCR tube containing reagents adapted to amplify the specific nucleic acid sequence if present in the tissue section, said reagents including first primers adapted to amplify the specific nucleic acid sequence and second primers adapted to amplify a nucleic acid sequence other than the specific sequence and located substantially throughout the tissue section, the ratio of first:second primers being 5:1 or greater;
(d) amplifying the specific nucleic acid sequence and the other sequence by PCR;
(e) extracting an aliquot from the solution for use as a template for manufacture of a labelled probe; and
(f) detecting the specific nucleic acid sequence by in-situ hybridization with the labelled probe.

13. A method of making a labelled probe, for use in in-situ hybridisation, comprising

identifying a tissue section containing the probe sequence,

attaching said tissue section to a support surface to form a composite;

immersing said composite in solution containing PCR reagents; and

amplifying the probe sequence using PCR techniques to make labelled probe.

14. A method according to Claim 13 comprising amplifying the probe sequence in a PCR tube.

15. A kit for carrying out in-situ amplification of a target double stranded nucleic acid and identification thereof using in-situ hybridisation, the kit comprising:-

at least two PCR primers, adapted to be substantially complementary to respective ends of respective strands of the target nucleic acid sequence, and

a labelled nucleic acid (such as DNA) probe adapted to be substantially homologous with a portion of or the whole of a PCR product generated by the PCR primers.

16. A kit according to Claim 15 wherein the labelled probe is prepared according to the method of Claim 13 or 14.

FIG. 1.

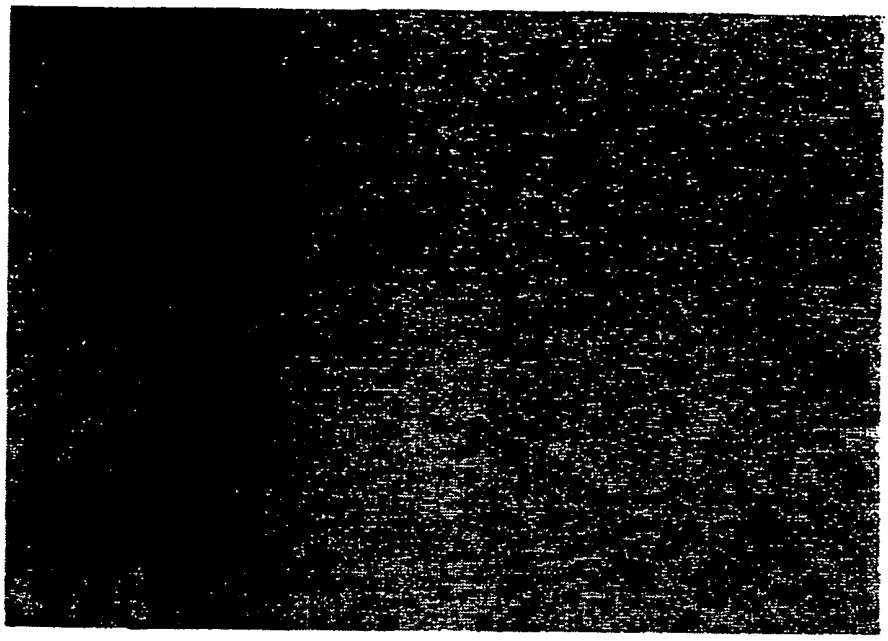


FIG. 2.

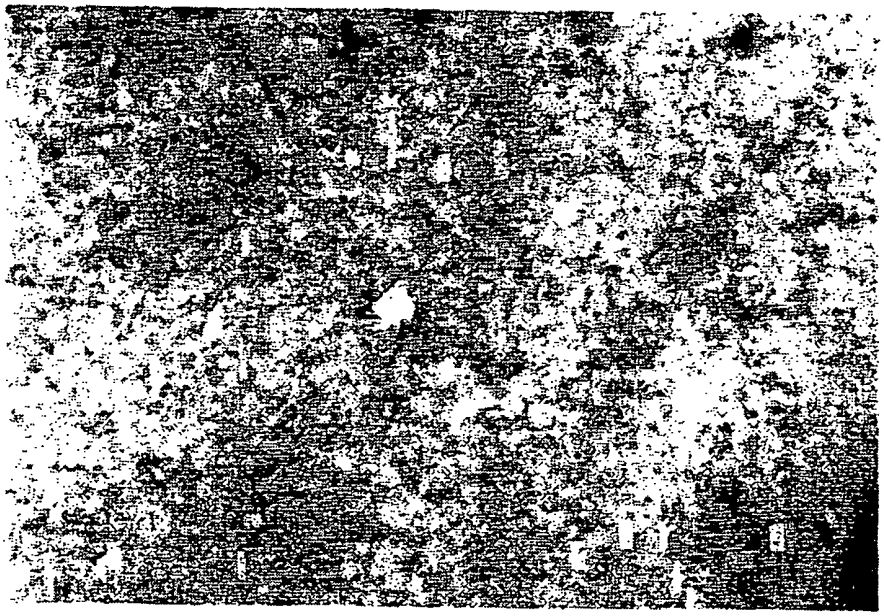


FIG. 3.

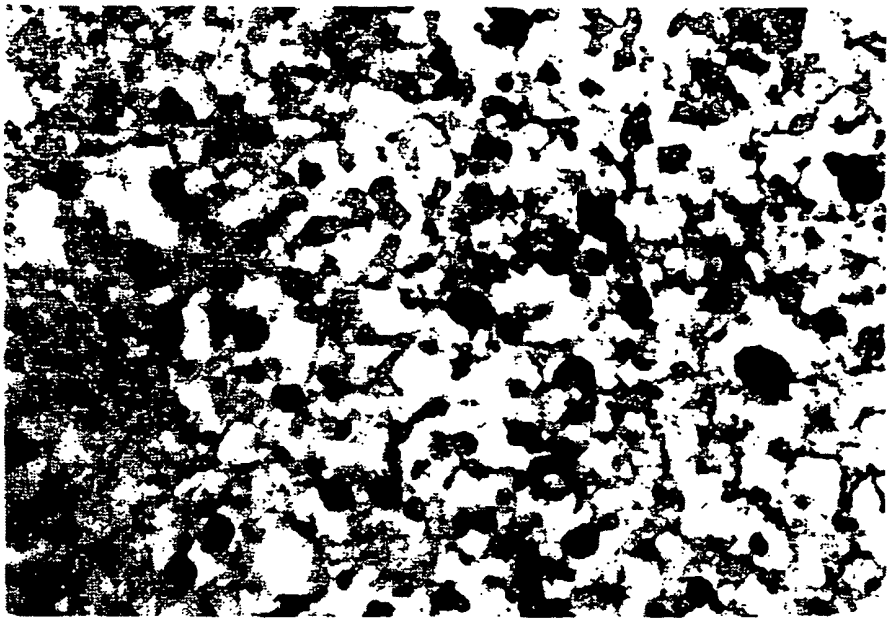


FIG. 4.

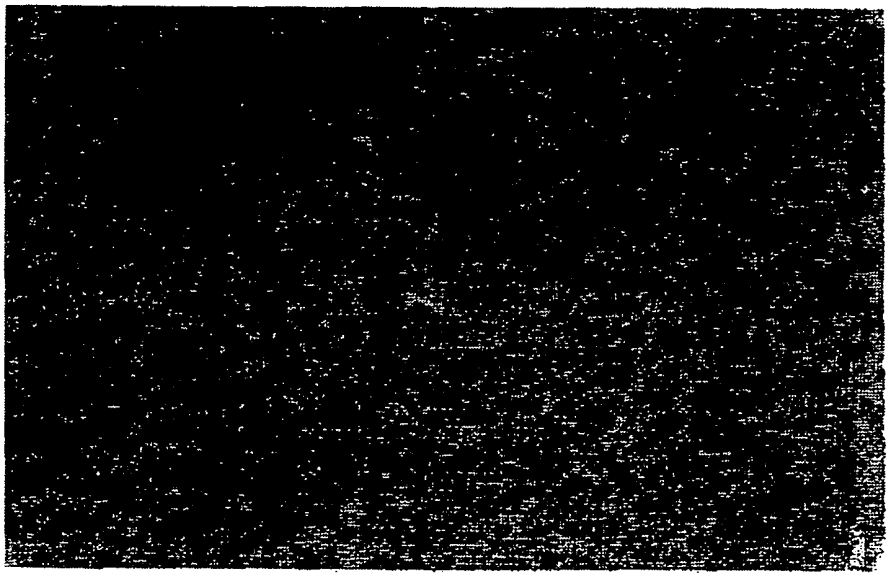


FIG. 5.

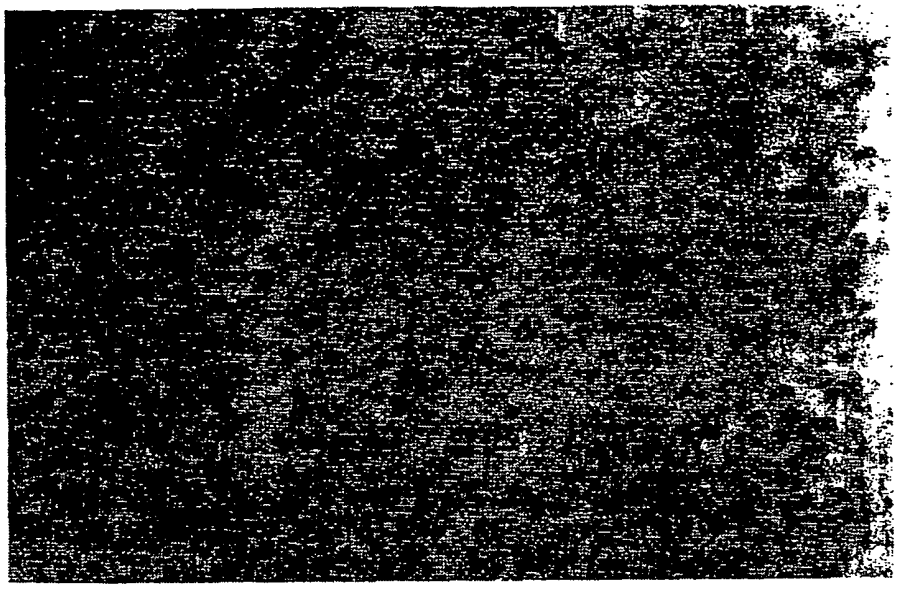


FIG. 6.

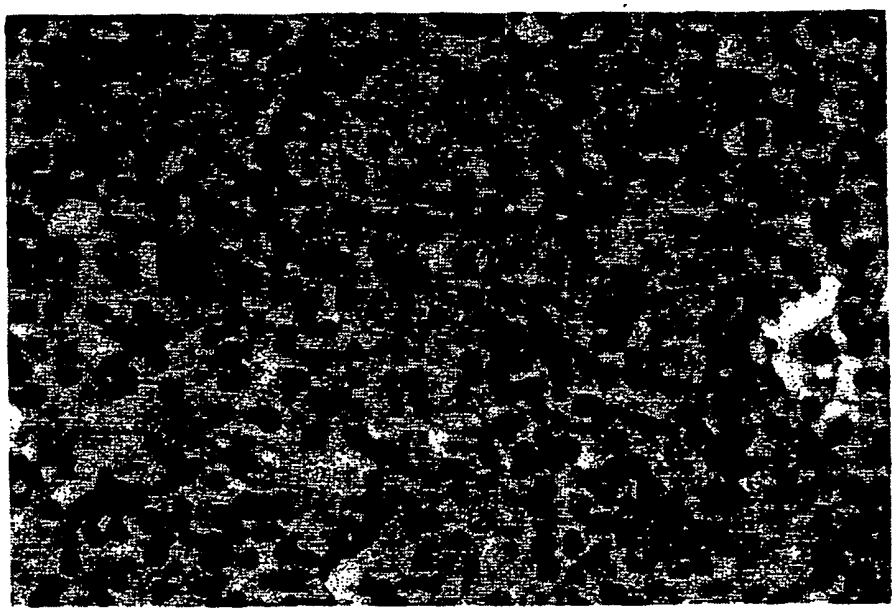


FIG. 7.

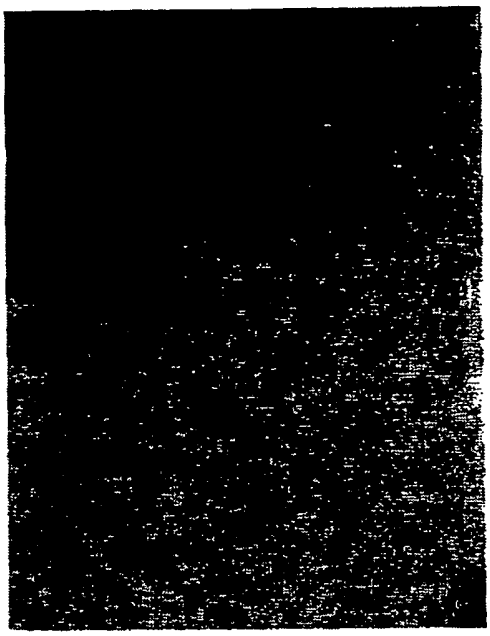


FIG. 8.

FIG. 9.

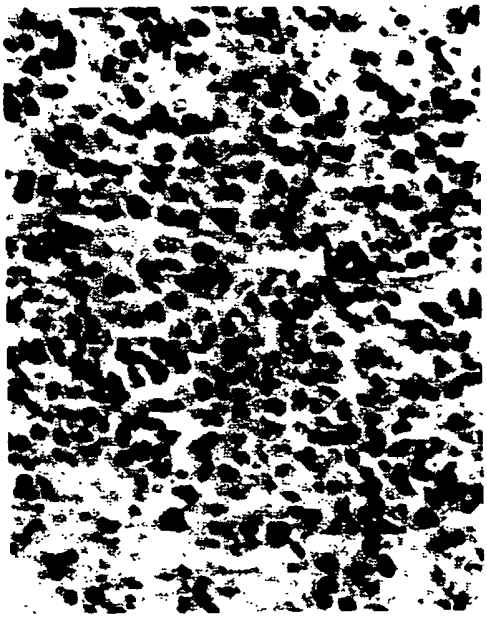


FIG. 10.

FIG. 11.

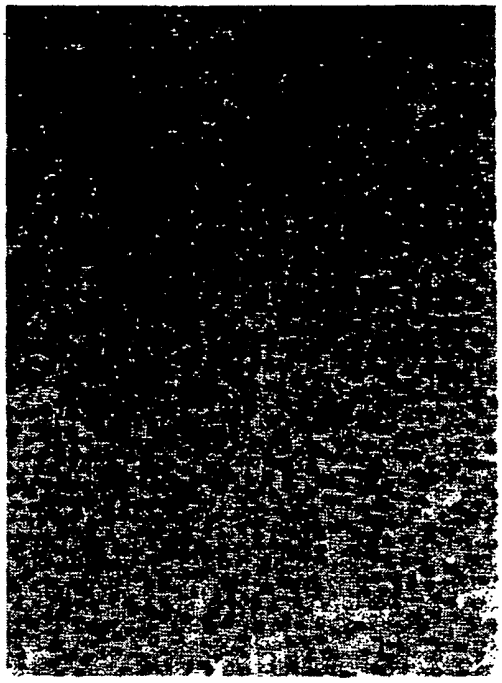


FIG. 12.

FIG. 13.

FIG. 14.

FIG. 15.

FIG. 16.

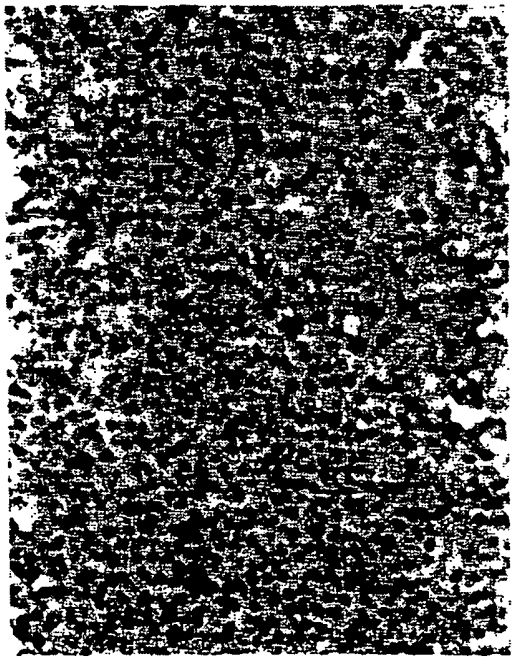
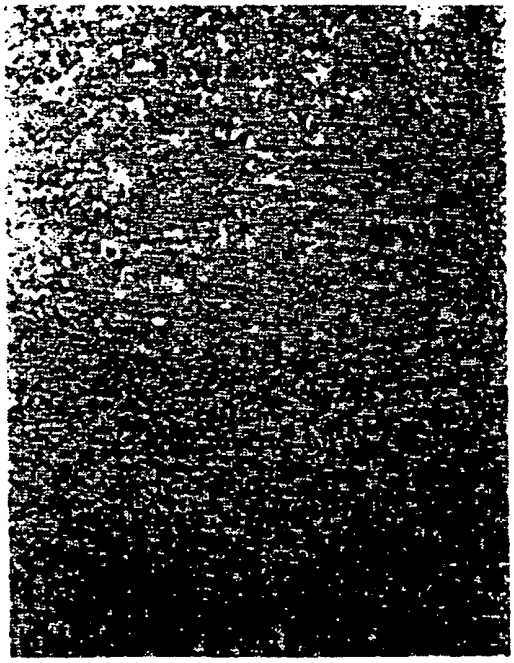


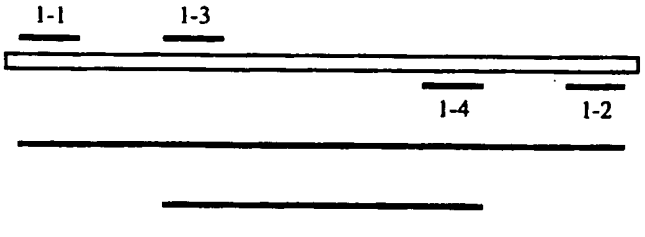
FIG. 17.

FIG. 18.

FIG. 19.



Case 1: EBV BamHI W

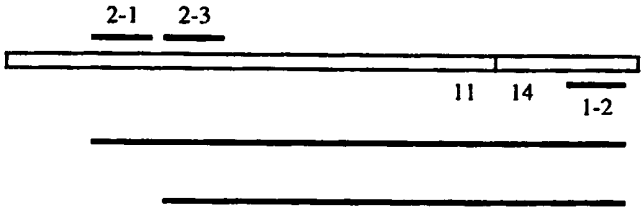


Template

IH-PCR 315 bp

Probe 200 bp

Case 2: t(11;14)

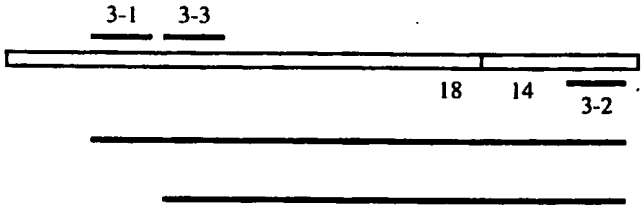


Template

IH-PCR 218 bp

Probe 198 bp

Case 3: t(14;18)

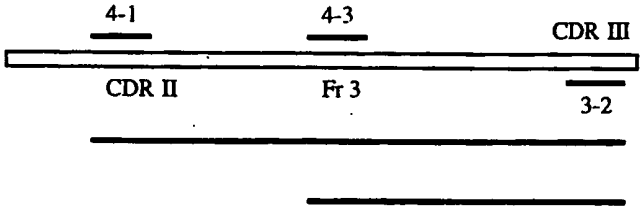


Template

IH-PCR 174 bp

Probe 154 bp

Case 4: IgVH



Template

IH-PCR 176 bp

Probe 93 bp

FIG. 20.

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/GB 96/01981

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 158, no. 1, 1 January 1993, AMSTERDAM, pages 131-145, XP000332064 BAGASRA O ET AL: "POLYMERASE CHAIN REACTION IN SITU: INTRACELLULAR AMPLIFICATION AND DETECTION OF HIV-1 PROVIRAL DNA AND OTHER SPECIFIC GENES" see page 133 - page 134; figure 1 ---	1
A	EP,A,0 611 598 (PERKIN ELMER CORP) 24 August 1994 cited in the application see abstract; figures ---	1
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 November 1996	17. 12. 96

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/GB 96/01981

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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